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(54) Title: METHOD FOR SCREENING FOR SUBSTANCES WHICH ARE ACTIVATORS OR INHIBITORS OF PROTEIN KINASE B

(57) Abstract

The present invention relates to methods for screening for substances which are activators or inhibitors of Protein kinase B (PKB) and can be used as a kinase substrate for PKB by the use of peptides comprising a specific sequence which do not include a large hydrophobic residue at the C-terminal end. These peptides can be used in assays measuring the activity of PKB, in screening for substances which are activators or inhibitors of gene transcriptional regulation of forkhead proteins through the catalytic activities of PKB and for discrimination between the effects of compounds which mediate insulin action through transcription from those which modulate activity of enzymes involved in metabolism by phosphorylation. The substrate peptide sequence comprises the sequence 1 ArgXaaArgXaaXaaSerXaa sequence 2, in which, Xaa in position 2 is any amino acid, preferably chosen from Pro and Gly, Xaa in positions 4 and 5 are any amino acid, peferably chosen from Thr and Ser and Xaa in position 7 is any amino acid, preferably chosen from Asn, Gln, Thr, Ser and with the provisi that the sequence does not include a large hydrophobic residue directly C-terminal to the phosphorylation site.

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Method for screening for substances which are activators or inhibitors of Protein kinase B

Summary

The present invention relates to method for screening for substances which are activators or inhibitors of Protein kinase B (PKB) and can be used as a kinase substrate for PKB by the use of peptides comprising a specific sequence which do not include a large hydrophobic residue at the C-terminal end. These peptides can be used in assays measuring the activity of PKB, in screening for substances which are activators or inhibitors of gene transcriptional regulation of forkhead proteins through the catalytic activities of PKB and for discrimination between the effects of compounds which mediate insulin action through transcription from those which modulate activity of enzymes involved in metabolism by phosphorylation.
The substrate peptide sequence comprises the sequence 1 ArgXaaArgXaaXaaSerXaa or sequence 2, ArgXaaArgXaaXaaThrXaa, in which Xaa in position 2 is any amino acid, preferably chosen from Pro and Gly, Xaa in positions 4 and 5 are any amino acid, preferably chosen from Thr and Ser and Xaa in position 7 is any amino acid, preferably chosen from Asn, Gln, Thr, Ser and with the proviso that the sequence does not include a large hydrophobic residue directly C-terminal to the phophorylation site.

Background

The mechanistic basis by which insulin regulates the disposition of glucose by animals has been elucidated in close detail in recent years. A cascade of interacting proteins has been described which, when functioning in the normal situation, serve to transduce the signals emanating from insulin, causing cells of different origin to take up glucose from the bloodstream and store it (White 1997). In the current understanding, activation of the insulin receptor by insulin causes the phosphorylation and activation of insulin receptor substrate (IRS) proteins. These serve to act as docking proteins for a

variety of downstream proteins leading to their activation. A key downstream protein in insulin signalling is phosphoinositide 3-kinase (PI3K) which catalyses the production of the second messenger phosphatidylinositol 3,4,5-trisphosphate. This is a lipid and which is central to the activation of PKB ((Franke et al. 1995); (James et al. 1996); (Franke et al. 1997); (Klippel et al. 1997); (Alessi et al. 1997); (Stokoe et al. 1997)). It is bound by the pleckstrin homology (PH) domains of PKB and of an upstream kinase called 3-phosphoinositide-dependent kinase 1 (PDK1) which is involved in the activation of PKB.

PKB appears to be a key intermediary in the regulation of glucose utilisation and control of protein synthesis by insulin (Cross et al. 1995); (Cohen et al. 1997); (Peak et al. 1998); (Gingras et al. 1998)). Thus, it has been demonstrated to phosphorylate and inactivate glycogen synthase kinase 3 (GSK3; (Cross et al. 1995)), permitting the synthesis of glycogen from glucose. Furthermore, in cardiac myocytes, PKB has been shown to phosphorylate and activate phosphofructo kinase-2 (Deprezet al. 1997) whose product, fructose 2,6-bisphosphate, acts as an allosteric activator of glycolysis. A third likely substrate for PKB is the type 3B cyclic AMP phosphodiesterase (Wijkander et al. 1998), which in insulin-responsive tissues is activated by phosphorylation, leading to the inactivation of adrenergic-stimulated processes.

Although the range of substrates phosphorylated by PKB is diverse, all of those described share a common short primary sequence which serves as the target region for PKB. The first identified consensus sequence of amino acids was in GSK-3, described as GlyArgProArgThrSerSerPheAlaGluGly (GRPRTSSFAEG) (Cross et al. 1995). See also WO 97/22360.

Comparison with other substrates allows a consensus sequence to be derived from this which is likely to contain the essential features for phosphorylation by PKB. This consensus is: RXRXXS/TF (Alessi et al. 1996) where F is phenylalanine but could be replaced by another bulky hydrophobic residue. Data in the literature (not only Alessi et al. 1996 but also Walker et al. 1998) strongly suggest that the amino acid sequence for phophorylation by PKB must include a large hydrophobic residue directly C-terminal to the phophorylation site.

Such a residue has thus been described as crucial for phosphorylation by PKB.

Studies in *C. elegans* have recently suggested that an important effect of insulin may be to suppress the transcriptional activity in the family of transcription factors (Ogg et al. 1997). By inactivating a specific transcription factor in *C. elegans* the authors have shown that *C. elegans* can recover metabolism in the absence of the insulin receptor.

Figures

Figure 1a: Cos-7 transfected with HA-PKB. Phosphorylation of Crosstide (black bar) compared with a peptide according to the invention (grey bar)

Figure 1b: Inactive rPKBα activated by incubation with IGF-1 stimulated muscle cell lysate. Phosphorylation of Crosstide (black bar) compared with a peptide according to the invention (grey bar)

Figure 2a. Phosphorylation of peptides compared with that of the peptide sequence RPRTSSF.

Figure 2b. Phosphorylation of peptides with different modifications compared with that of the peptide sequence GRPRTSSF.

The invention

We have now found peptides which do not include a large hydrophobic residue directly C-terminal to the phophorylation site.

These peptides have unexpectedly been shown to be as good substrates for PKB as the earlier known peptides with the large hydrophobic residue directly C-terminal to the phophorylation site.

These novel peptides can be used in screening for substances which are activators, inhibitors or binders of Protein kinase B (PKB).

The invention is defined in the attached claims.

The peptides can be used for discrimination between the effects of compounds which mediate insulin action through transcriptional regulation from those compounds which modulate activity of enzymes involved in metabolism by phosphorylation. Such a discrimination has not been possible earlier.

The invention relates to the use of a peptide sequence comprising the sequences ArgXaaArgXaaXaaSerXaa, sequence 1, or ArgXaaArgXaaXaaThrXaa, sequence 2. The sequence can be included as a part of any peptide or protein provided that the sequence is accessible to the targeting enzyme PKB.

The peptide is preferably SerThrPheArgProArgThrSerSerAsnAla, sequence 14. (STFRPRTSSNA).

The sequences used in the screening method are defined in the attached claims.

The amino acids Asp, Glu, Lys and Arg are charged and could possibly have an influence on phosphorylation.

By large or strongly hydrophobic residue is for example meant Phe, Leu, Ile, Trp and Cys.

By PKB any isoform thereof is included.

The uses of the defined sequences are defined in the attached claims.

Gene transcriptional regulation involves activation or repression of the enzymes and other components involved in metabolism, for example the repression of PEPCK (phosphoenol pyruvate carboxykinase) in liver.

Members of the forkhead transcription factor family can be exemplified by FKHR, FKHRL1, AFX and AF6q21 (Accession number AF032885, AF032886, X939996 and AJ001589, respectively).

Furthermore the peptides as defined in the claims can be used in the search for new substrates for PKB as templates for sequence searches and/or for primers in techniques of molecular biology such as PCR.

Example 1

The α isoform of PKB was immunoprecipitated from lysates of Cos-7 cells (approx. 1mg of protein) transfected with bovine PKBα which contained an N-terminal haemagglutinin (HA) tag. After washing, phosphorylation of the peptides RPRTSSF (black bar in Figure 1a) and STFRPRTSSNA (grey bar in Figure 1a) by PKB was measured in parallel assays by incubation in the presence of ^{33}P-labelled ATP.

Comparison of results from the two peptides showed that within a 30 minute assay, equal amounts of peptide were converted to the phosphorylated form at a rate of 0.5pmol per minute under the assay conditions employed.

As immunoprecipitation of PKB can co-precipitate other forms of the enzyme and COS7 cells contain wildtype PKB, the current assay will comprise other isoforms than PKBα.

Hence, the example suggest that the peptide of the invention is useful for determining PKB activity of other isoforms than the α-form.

Example 2

Recombinant, virtually inactive GST-PKBα, pre-coupled to glutathione beads was incubated and activated with increasing concentrations of lysate from IGF-1-stimulated H9C2 cardiac myocytes (approx. 0, 20, 100 and 200μg/ml) in the presence of unlabelled ATP. The beads were subsequently washed thoroughly and used to phosphorylate each of the peptides RPRTSSF (black bar in Figure 1b) and STFRPRTSSNA (grey bar in Figure 1b) in the presence of ^{33}P-labelled ATP. Data showed that each peptide was equivalently phosphorylated by PKB and that each peptide was phosphorylated to the same extent as the degree of activation of the enzyme increased.

This experiment proves that the peptide of the invention is effective for assaying activity of the PKBα isoform.

Example 3

The general features of peptide substrates for use in PKB phosphorylation assays were refined by investigating the ability of recombinant activated PKB to phosphorylate various different peptides (Figures 2a and b). Thus, peptides (100μM) were incubated with 0.2μg activated recombinant PKB (purchased from UBI) in the presence of 10μM ^{33}P-ATP and the extent of phosphorylation was compared with that of the peptide sequence RPRTSSF, previously used as a substrate for PKB (Alessi *et al*, 1996). Figure 2a shows that addition of up to three neutral or small hydrophobic amino acids to either end of the sequence PKB is thought to phosphorylate had no marked effect on the extent of phosphorylation of each respective peptide. These data further reinforce our assertion that peptides derived from the fork head transcription factor family of proteins which lack a bulky hydrophobic amino acid C-terminal to the phosphorylated residue are good substrates for PKB.

Figure 2b shows the ability of PKB to phosphorylate peptides with different modifications. Addition of a bulky unit such as a biotin moiety to the N-terminal end of the peptide GRPRTSSF reduced phosphorylation by PKB by 75%. In addition, peptides KKRNRTLTK (a peptide often used to measure the activity of a kinase related to PKB, p70 S6 kinase) and APRPRVETSQ (derived from pyruvate dehydrogenase kinase 1) were phosphorylated to less than one quarter the extent of GRPRTSSF by PKB. These data indicate that addition of charged amino acids to either end of the consensus peptide sequence phosphorylated by PKB markedly reduces the ability of PKB to phosphorylate them. In particular, each peptide contains a charged residue immediately adjacent to the amino acid which becomes phosphorylated, indicating that PKB may favour no charge at these positions, directly N- and C-terminal to the targetted residue.

Discussion.

Our data show that the reported requirement for a large hydrophobic amino acid residue directly C-terminal to the targetted residue for PKB-mediated phosphorylation is not necessary for an efficient phosphorylation, and that most amino acids are suitable in the same position (as seen in FKHR and other members of the transcription factor family) without compromising the ability of PKB to phosphorylate.

The data also show that our claimed novel peptides can be used as an efficient component in the screening for PKB modulators.

The claimed peptides can be used for finding compounds useful for treating patients having a deficiency of the amount of essential components in metabolism such as transducers of the insulin signalling pathway and enzymes involved in metabolism. The compounds found from the claimed screening are anticipated also to be used against long term complications resulting from insulin resistance, such as vascular dysfunction, loss of neuronal cells and β-cells in pancreas. These compounds are not possible to find by using the modulators as described in WO 97/22360.

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CLAIMS

1. Method for screening for substances which are activators or inhibitors of Protein kinase B (PKB) by the use of a substrate peptide comprising the sequences 1 or 2

ArgXaaArgXaaXaaSerXaa (1) or ArgXaaArgXaaXaaThrXaa (2)

in which

Xaa in position 2 is any amino acid, preferably chosen from Pro and Gly,

Xaa in positions 4 and 5 are any amino acid, preferably chosen from Thr and Ser and

Xaa in position 7 is any amino acid, preferably chosen from Asn, Gln, Thr, Ser

and with the proviso that the sequence does not include a large hydrophobic residue directly C-terminal to the phophorylation site.

2. Method for screening according to claim 1 with the proviso that

Xaa in positions 4 and 5 is not chosen from Asp, Glu, Lys or Arg.

3. Method for screening according to claim 1 or 2 in which the peptide consists of the primary sequence 3

ArgProArgThrSerSerAsn (3)

4. Method for screening according to claim 1 in which the peptide comprises the sequence 4 or 5

XaaArgXaaArgXaaXaaSerXaa (4) or XaaArgXaaArgXaaXaaThrXaa (5)

in which

Xaa in position 1 is a large hydrophobic amino acid, preferably Phe.

Xaa in position 3 is any amino acid, preferably chosen from Pro and Gly,

Xaa in positions 5 and 6 are any amino acid, preferably chosen from Thr and Ser and

Xaa in position 8 is any amino acid, preferably chosen from Asn, Gln, Thr, Ser

and with the proviso that the sequence does not include a large hydrophobic residue directly C-terminal to the phophorylation site.

5. Method for screening according to claim 4 with the proviso that Xaa in positions 5 and 6 is not chosen from Asp, Glu, Lys or Arg.

6. Method for screening according to any of claims 1 to 3 in which the peptide comprises the sequence 6 or 7

ArgXaaArgXaaXaaSerXaaXaa (6) or ArgXaaArgXaaXaaThrXaaXaa (7)

in which

Xaa in position 8 is a small hydrophobic amino acid, preferably chosen among Ala and Gly.

7. Method for screening according to any of claims 4 to 6 in which the peptide comprises the sequence 8 or 9

XaaArgXaaArgXaaXaaSerXaaXaa (8) or XaaArgXaaArgXaaXaaThrXaaXaa (9)

in which

Xaa in position 9 is a small hydrophobic amino acid, preferably chosen among Ala and Gly.

8. Method for screening according to claim 7 in which the peptide comprises the sequence 10 or 11

PheArgXaaArgXaaXaaSerXaaXaa (10) or PheArgXaaArgXaaXaaThrXaaXaa (11)

9. Method for screening according to claim 7 in which the peptide comprises the sequence 12

SerThrPheArgXaaArgXaaXaaSerXaaXaa (12)

in which

Xaa in position 5 is any amino acid, preferably chosen from Pro and Gly,

Xaa in positions 7 and 8 are any amino acid, preferably chosen from Thr and Ser and

Xaa in position 10 is any amino acid, preferably chosen from Asn, Gln, Thr, Ser

Xaa in position 11 is a small hydrophobic amino acid, preferably chosen among Ala and Gly.

10. Method for screening according to claim 8 in which the peptide comprises the primary sequence 13

SerThrPheArgXaaArgXaaXaaThrXaaXaa (13)

in which
Xaa in position 5 is any amino acid, preferably chosen from Pro and Gly,
Xaa in positions 7 and 8 are any amino acid, preferably chosen from Thr and Ser and
Xaa in position 10 is any amino acid, preferably chosen from Asn, Gln, Thr, Ser
Xaa in position 11 is a small hydrophobic amino acid, preferably chosen among Ala and Gly.

11. Method for screening according to claim 9 in which the peptide consists of the primary sequence 14

SerThrPheArgProArgThrSerSerAsnAla (14)

12. Method for screening for substances which are activators, inhibitors and binders of gene transcriptional regulation by forkhead proteins through the catalytic activities of PKB characterised by the use of any of the peptides defined in any of claims 1-11.

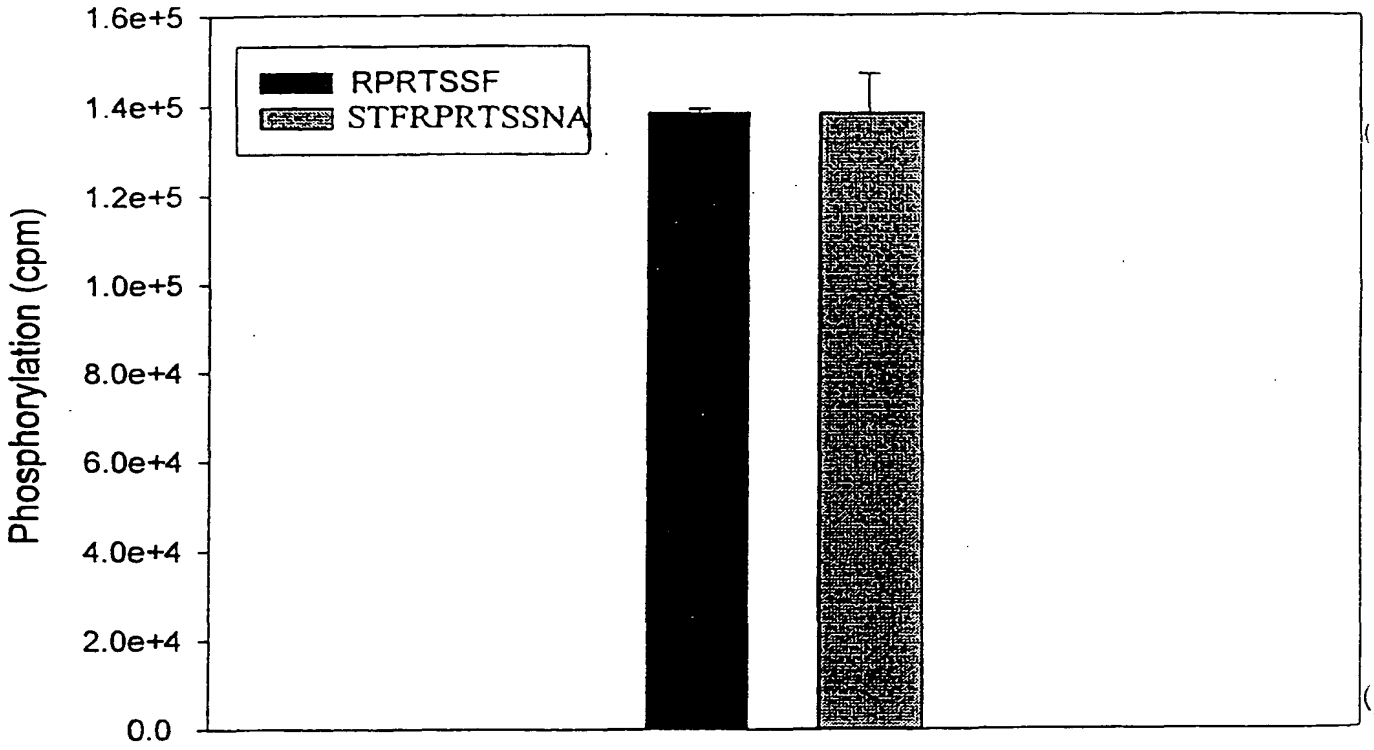
13. Method for screening for substances which have the capacity to modulate PKB activity characterised by the use of any of the peptides defined in any of claims 1-11.

14. Use of a peptide as defined in any of claims 1-11 in assays measuring the activity of PKB.

15. Use of a peptide as defined in any of claims 1-11 in screening for substances which are activators or inhibitors of gene transcriptional regulation by forkhead proteins through the catalytic activities of PKB.

16. Use of the peptide as defined in any of claims 1-11 for discrimination between the effects of compounds which mediate insulin action through transcription from those which modulate activity of enzymes involved in metabolism by phosphorylation.

17. Use according to claim 14 for discrimination between the effects of compounds which mediate insulin action through transcription via forkhead transcription factor family from those which modulate activity of enzymes involved in metabolism by phosphorylation.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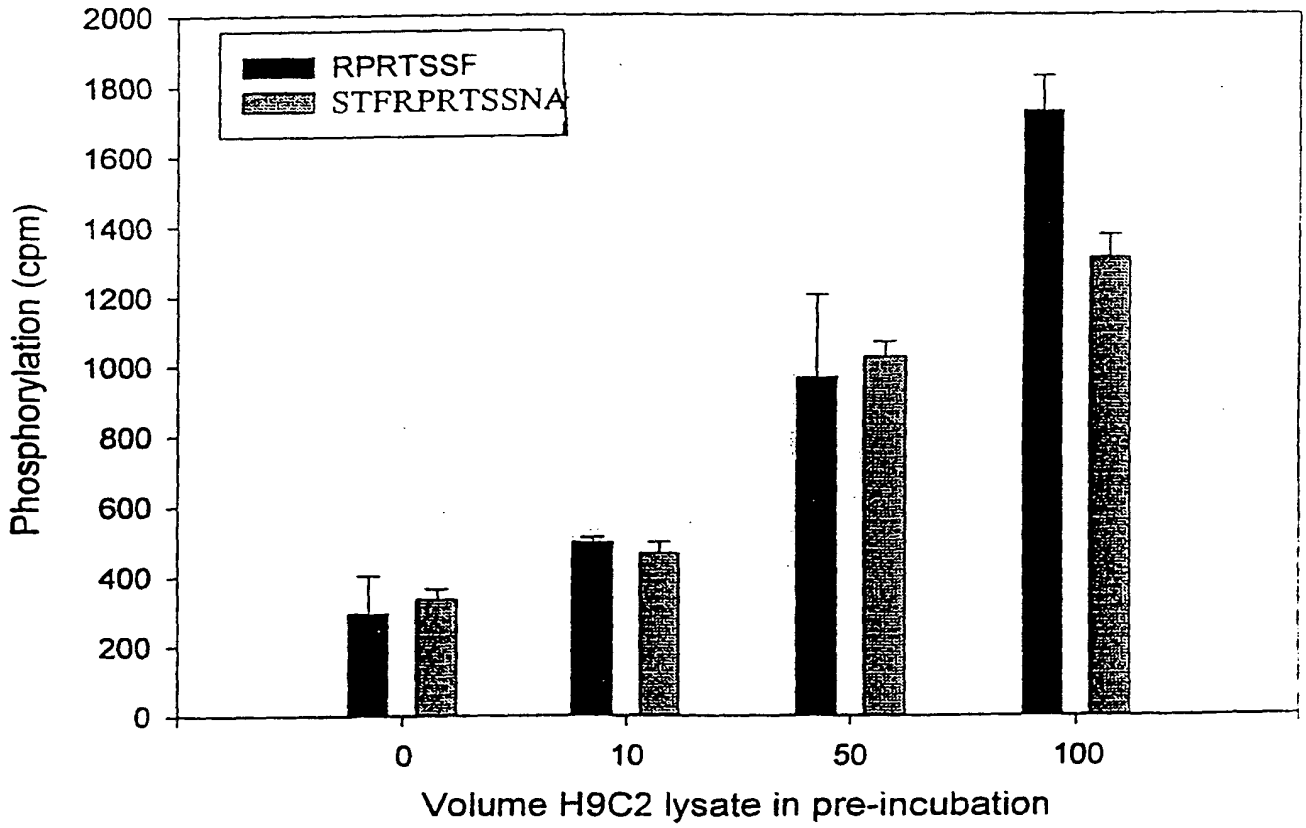
STFRPRTSSNA

Figure 1a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Volume H9C2 lysate in pre-incubation

Figure 1b

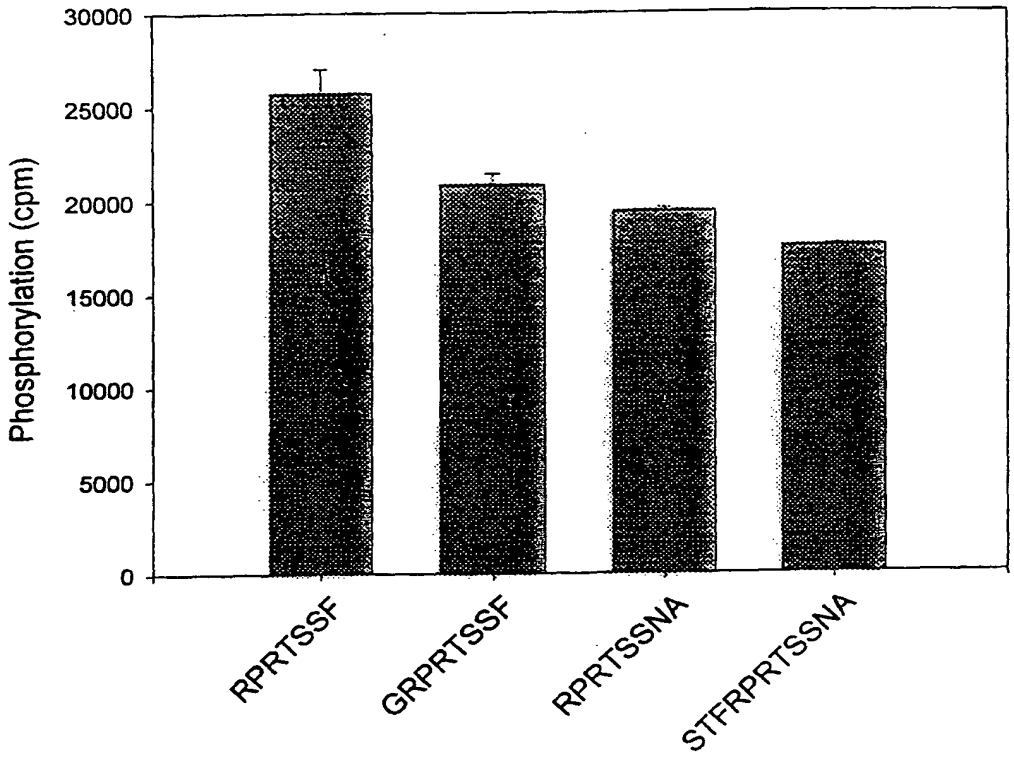
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Figure 2a

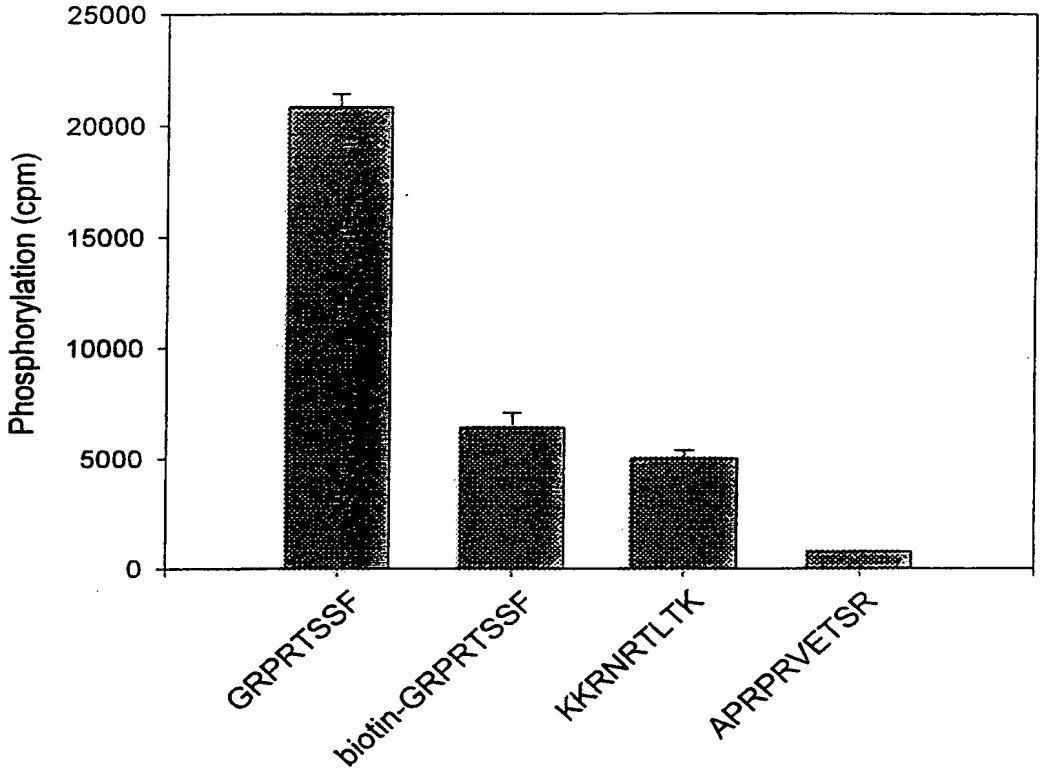
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Figure 2b

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/00609

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/48
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, REGISTRY, MEDLINE, BIOSIS, EMBASE, DBA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS Letters, Volume 399, 1996, Dario R. Alessi et al, "Molecular basis for the substrate specifity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase", page 333 - page 338, see table 1 --	1-17
A	WO 9722360 A2 (MEDICAL RESEARCH COUNCIL), 26 June 1997 (26.06.97), page 39, line 13 - page 41, line 8 --	1-17
A	WO 9518823 A2 (BETH ISRAEL HOSPITAL), 13 July 1995 (13.07.95), page 18, line 6 - line 13; page 30, line 26 - page 32, line 40 --	1-17

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Date of the actual completion of the international search	Date of mailing of the international search report
11 August 1999	26 -08- 1999
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/00609

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